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(54) Title: METHOD OF TREATING BLADDER AND LOWER URINARY TRACT SYNDROMES

(57) Abstract

The present invention relates to bladder and lower urinary tract syndromes, particularly, irrative symptoms, and to a method of treating same using α_{1d}-adrenergic receptor (α_{1d}AR) antagonists. The invention further relates to a method of screening compounds for their ability to serve as α_{1d}AR selective antagonists.

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METHOD OF TREATING BLADDER AND LOWER URINARY TRACT SYNDROMES

This application claims priority from US Provisional Application Number 60/084,479. filed May 6. 1998, the entire contents of that application being incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to bladder and lower urinary tract syndromes, particularly, irritative symptoms, and to a method of treating same using α_{1d}-adrenergic receptor (α_{1d}AR) antagonists. The invention further relates to a method of screening compounds for their ability to serve as α_{1d}AR antagonists.

BACKGROUND

Lower urinary tract symptoms (LUTS) resulting from bladder outlet obstruction (BOO) remains one of the most commonly encountered disorders in urology, and can be secondary to fixed anatomical and/or functional causes (Steers et al, Voiding dysfunction: diagnosis, classification, and management, in Adult and Pediatric Urology; Third Edition, J.Y. Gillenwater, et al., Editors. 1996. Mosby-Year Book, Inc.: St. Louis. p. 1220-1325.). Causes of BOO include prostatic enlargement (benign or malignant), bladder neck contracture, urethral stricture, and meatal stricture (Steers et al. Voiding dysfunction: diagnosis, classification, and management, in Adult and Pediatric Urology; Third Edition, J.Y. Gillenwater, et al., Editors. 1996, Mosby-Year Book, Inc.: St. Louis. p. 1220-1325.). Symptoms associated with BOO typically fall into obstructive or irritative categories; obstructive symptoms include hesitancy, poor stream, prolonged urination, and feelings of incomplete emptying, while irritative symptoms consist

of frequency, urgency, nocturia, and unstable bladder contractions. The bladder is functionally and anatomically divided into the detrusor (body and ventral base) and trigone (dorsal portion of base extending between the ureteral orifices and the bladder neck) (Zderic et al, Voiding function: relevant anatomy, physiology, pharmacology, and molecular aspects, in Adult and Pediatric Urology; Third Edition, J.Y. Gillenwater, et al., Editors. 1996, Mosby-Year Book, Inc.: St. Louis. p. 1159-1219), with distinct histology, histochemistry, and pharmacology. In contrast, the prostate and trigone have similar vascular supply, innervation, and receptor expression (Gosling et al, Detrusor morphology in relation to bladder outflow obstruction and instability, in Benign Prostatic Hypertrophy, F. Hinman, Editor. 1983, Springer-Verlag: Berlin. p. 666-71).

The physiology of LUTS secondary to benign prostatic hypertrophy (BPH) has two components: (1) a static component related to the increase in prostatic cellular mass and (2) a dynamic component related to variations in prostatic smooth muscle tone (Caine et al, Brit. J. Urol. 47:193-202 (1975)). Histologically BPH is characterized by glandular (epithelial) and stromal (fibromuscular) hyperplasia, with the latter being the dominant factor in the pathogenesis of clinically significant BPH (Shapiro et al, J. Urol. 147:1293-1297 (1992)). Therefore much attention has focused on the role of the sympathetic nervous system and α_1-adrenergic receptors (α_1ARs) in the dynamic component of BOO, leading to clinical studies of α_1AR antagonists as agents to relieve outlet obstruction. These studies have found that α_1AR antagonists relax prostatic smooth muscle, relieving obstructive symptoms (Chapple, Brit. J. Urol. 1:47-55 (1995), Caine, Urol. Clin. N. Am. 17:641-649 (1990), Kawabe and Niijima, Urol. Int. 42:280-284 (1987), Lepor et al, J. Urol. 148:1467-1474 (1992), Reuther and

Aagaard, Urol. Int. 39:312-313 (1984), Matyus and Horvath, Med. Res. Rev. 17:523-535 (1997)). In addition, α_1AR antagonists have been found to relieve the irritative bladder symptoms in men (most often associated with BPH) and women (Matyus and Horvath, Med. Res. Rev. 17:523-535 (1997), Serels and Stein, Neurourol. Urodyn. 17:31-36 (1998)). While it is logical to assume that elimination of BOO would relieve irritative symptoms, a number of recent studies suggest that the relationship between bladder irritability and outlet obstruction is not straightforward (Caine, Urol. Clin. N. Am. 17:641-649 (1990), Chapple and Smith, Brit. J. Urol. 73:117-123 (1994), Steers and De, J. Urol. 140:864-71 (1988), Steers et al, Am. J. Physiol. 266:R20 (1994)).

α_1ARs are members of the larger family of G protein-coupled adrenergic receptors which mediate actions of the endogenous catecholamines norepinephrine (NE) and epinephrine, resulting in smooth muscle contraction. cDNAs encoding three distinct α_1AR subtypes (α_{1a}, α_{1b}, α_{1d}) have been cloned, expressed in cells, and resultant protein characterized pharmacologically (Schwinn et al, J. Pharmacol. Exper. Ther. 272:134-142 (1995), Hieble et al, Pharmacol. Rev. 47:267-70 (1995)). α_{1a}ARs predominate in prostate and bladder trigone (Price et al, J. Urol. 150:546-551 (1993)), and have been shown to be functionally important in mediating prostate smooth muscle contraction (Forray et al, Mol. Pharmacol. 45:703-708 (1994), Lepor et al., J. Pharmacol. Exper. Ther. 270:722-727 (1994)). In addition to the three cloned α_1AR subtypes which have high affinity for the antagonist prazosin, a fourth type of α_1AR with low affinity for prazosin (α_{1L}) has been postulated (Muramatsu et al, Brit. J. Urol. 74:572-578 (1994)). In spite of initial evidence suggesting a role for the α_{1L}AR in human prostate smooth muscle contraction (Ford et al, Mol. Pharmacol. 49:209-215 (1996)), more recent data suggests RS17053 (the compound used in these studies)

detects a low affinity state of the α_{1a}AR in tissues rather than a distinct α_{1L}AR (Ford et al, Br. J. Pharmacol. 121:1127-1135 (1997)). Since non-selective α_1AR antagonists currently used to treat BPH have undesirable side-effects including light headedness, dizziness, and asthenia (Carruthers, Drug Safety 11:12-20 (1994)), many investigators have suggested that α_{1a}AR subtype selective antagonists might be beneficial in improving BPH-related symptoms via relieving BOO (Matyus and Horvath, Med. Res. Rev. 17:523-535 (1997), Hieble and Ruffolo, Jr., Exp. Opin. Invest. Drugs 6:367-387 (1997)). However, this approach does not take into account that irritative symptoms may persist in spite of relief of outlet obstruction (Hieble and Ruffolo, Jr., Exp. Opin. Invest. Drugs 6:367-387 (1997)).

Very little information exists regarding the role of α_1ARs in human detrusor. One of the few studies addressing this issue suggests human bladder (dome) contains only α_{1a}ARs (Walden et al, J. Urol. 157:1032-1038 (1997)). However, since irritative bladder symptoms persist in some patients despite relief of BOO, nonselective α_1AR antagonists may relieve the irritative effects of BPH through direct effects on bladder detrusor or other sites involved in micturation. The present invention results from the realization that human detrusor expresses two α_1AR subtypes (α_{1d}>α_{1a}). This realization makes possible the identification of α_1AR subtype selective antagonists that can be used to treat irritative symptoms.

SUMMARY OF THE INVENTION

The present invention relates generally to bladder and lower urinary tract syndromes and, more particularly, to a method of identifying α_{1d}AR antagonists that can be used to treat irritative symptoms. The invention also relates to a method of treating irritative symptoms using such agents.

Objects and advantages of the invention will be apparent from the detailed description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1A-1C. Schematic of the location of α_1AR subtype probes. Highlighted in bold are regions of α_{1a}(Fig. 1A), α_{1b}(Fig. 1B), and α_{1d}(Fig. 1C) ARs encoded by probes used in RNase protection assays.

FIGURE 2. Representative saturation binding isotherm generated using increasing concentrations of the α_1AR radiolabeled antagonist [^{125}I]HEAT in human detrusor membranes. Kd is 130±1.09 pM (n=5), similar to that reported for cells stably expressing each cloned human α_1AR subtype (Schwinn et al, J Pharmacol. Exper. Ther. 272:134-142 (1995) - $\alpha_{1a/d}$AR of the reference refers to the α_{1d}AR subtype described herein since the α_1AR nomenclature used here is the IUPHAR nomenclature (Hieble et al, Phar. Rev. 97:267 (1995)).

FIGURE 3. RNase protection assays examining α_1AR subtype expression in detrusor were performed in all patients (n=13). A representative RNase protection assay showing results from five patients is shown. In this experiment, radiolabeled probe for each α_1AR subtype is shown at the far left along with (from left to right) protected fragments resulting from total RNA extracted from rat-1 fibroblast cells stably expressing each cloned human α_1AR subtype (20 mg; positive probe control), yeast tRNA (20 mg; negative control); and total RNA isolated from human detrusor (20 mg) from five patients (lanes 1-5). Gel exposure times are 24 hrs for probe and positive control lanes and 72 hrs for tRNA and human detrusor samples. Although the α_{1d}AR subtype mRNA band is stronger than the α_{1a}AR protected fragment, the α_{1a}AR probe contains 73%

more radiolabeled αUTP compared with the α_{1d}; hence, after normalization for radioactive label incorporation, two-fold predominance of the α_{1d}AR subtype in human detrusor is apparent.

FIGURES 4A and 4B. Results from RT-PCR experiments on human detrusor (Fig. 4A) and rat whole bladder (Fig. 4B) RNA. α_1AR subtype specific cDNA in plasmid vectors served as positive controls.

FIGURE 5. α_1AR subtype expression in human detrusor was determined using competition analysis with the α_{1d}AR-subtype selective ligand BMY7378. Results from a representative curve are shown demonstrating a two-site fit with high affinity Ki corresponding to the α_{1d}AR.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the recognition of α_{1d}AR as the α_1AR subtype responsible for irritative symptoms associated with bladder and lower urinary tract diseases. The invention provides, in one embodiment, a method of selecting α_{1d}AR antagonists and, in a further embodiment, a method of treating irritative symptoms using α_{1d}AR antagonists. (The nomenclature used herein is the new nomenclature provided in Hieble et al, Phar. Rev. 97:267 (1995)).

The method of treatment to which the invention relates comprises administering to a patient suffering irritative symptoms an amount of an α_{1d}AR antagonist sufficient to relieve such symptoms. In accordance with the invention, irritative symptoms include excessive frequency of urination, urgency of urination, nocturia and unstable bladder contractions. Patients amenable to treatment include men and women, children and adults. In males, a preferred antagonist is both an α_{1a}AR and an α_{1d}AR antagonist. In females, preferred

antagonists are α_{1d}AR specific antagonists. The amount of the antagonist to be administered and the treatment regimen will vary with the antagonist, the patient and the effect sought. Optimum doses and regimens, however, can be readily determined by one skilled in the relevant art.

The present invention also relates to a method of screening compounds for their ability to bind primarily to α_{1d}AR and thereby to function, potentially, as α_{1d}AR antagonists Preferred α_{1d}AR selective antagonists show at least a two fold selectivity for α_{1d}AR relative to α_{1a}AR or α_{1b}AR. Binding assays of this embodiment invention include cell-free assays in which α_{1d}AR, or portion thereof (e.g. relevant transmembrane portion -- see, generally. Hwa et al, J. Biol. Chem. 271:7956 (1996)), is incubated with a test compound (proteinaceous or non-proteinaceous) which, advantageously, bears a detectable label (e.g., a radioactive or fluorescent label). Preparations of membranes that bear α_{1d}AR can be used in this assay, including commercially available preparations (e.g. the NEN multireceptor kit (NET 1034)). Following incubation, the α_{1d}AR, or portion thereof, free or bound to test compound, can be separated from unbound test compound using any of a variety of techniques (for example, the α_{1d}AR (or portion thereof) (e.g., associated with a membrane) can be bound to a solid support (e.g., a plate or a column) and washed free of unbound test compound). The amount of test compound bound to α_{1d}AR, or portion thereof, is then determined using a technique appropriate for detecting the label used (e.g., liquid scintillation counting in the case of a radiolabelled test compound). (See Schwinn et al, J. Pharm. Exp. Ther. 272:134 (1995).)

Binding assays of this embodiment can also take the form of cell-free competition binding assays. Such assays can be conducted as described in the Examples that follow (see particularly Example 2 (the test compound being

substituted for BMY 7378) - see also Schwinn et al, J. Pharm. Exp. Ther. 272:134 (1995)). Alternatively, α_{1d}AR, or portion thereof, can be incubated with a compound known to interact, specifically, with α_{1d}AR (e.g., BMY7378), which compound, advantageously, bears a detectable label (e.g., a radioactive or fluorescent label). A test compound (proteinaceous or non-proteinaceous) is added to the reaction and assayed for its ability to compete with the known (labeled) compound for binding to α_{1d}AR, or portion thereof. Free known (labeled) compound can be separated from bound known compound, and the amount of bound known compound determined to assess the ability of the test compound to compete. This assay can be formatted so as to facilitate screening of large numbers of test compounds by linking the α_{1d}AR, or portion thereof or, to a solid support so that it can be readily washed free of unbound reactants.

α_{1d}AR, or portion thereof, suitable for use in the cell-free assays described above can be isolated from natural sources (e.g., as membrane preparations derived from bladder, e.g., human bladder) or prepared recombinantly or chemically. The α_{1d}AR, or portion thereof, or can be prepared as a fusion protein using, for example, known recombinant techniques. Preferred fusion proteins include a HIS tag, a FLAG tag, a GFP tag or other tag (moiety) suitable for use in colorimetric assays. Typically, the non-α_{1d}AR moiety is present in the fusion protein N-terminal to the α_{1d}AR, or portion thereof domain, but it can also be C-terminal.

As indicated above, the α_{1d}AR, or portion thereof, can be present linked to a solid support, including a plastic or glass plate or bead, a chromatographic resin, a filter or a membrane. Methods of attachment of proteins, or membranes containing same, to such supports are well known in the art.

The binding assays of the invention also include cell-based assays in which α_{1d}AR, or portion thereof, is associated with the cell membrane of an intact cell. Cells suitable for use in such assays include cells that naturally express α_{1d}AR and cells that have been engineered to express, advantageously, over express, α_{1d}AR (or portion thereof). Advantageously, cells expressing human α_{1d}AR are used. Suitable cells are preferably eucaryotic, including mammalian (human and nonhuman) cells, insect cells and yeast cells.

Cells can be engineered to express α_{1d}AR (advantageously, human α_{1d}AR, or portion thereof) by introducing into a selected host (e.g. a eucaryotic host) an expression construct comprising a sequence encoding α_{1d}AR, or portion thereof, operably linked to a promoter. A variety of vectors and promoters can be used. (See Schwinn et al, J. Pharm. Exp. Ther. 272:134 (1995).) Introduction of the construct into the host can be effected using any of a variety of standard transfection/transformation protocols (see Molecular Biology, A Laboratory Manual, second edition, J. Sambrook, E.F. Fritsch and T. Maniatis, Cold Spring Harbor Press, 1989). Cells thus produced can be cultured using established culture techniques suitable for the involved host. Culture conditions can be optimized to ensure expression of the α_{1d}AR (or portion thereof) encoding sequence.

While for the cell-based binding assays it is appropriate that the α_{1d}AR (or portion thereof) be associated with the cell membrane, for other purposes the expression product can be secreted into the culture medium or present in the cell cytoplasm.

The cell-based binding assays of the invention can be carried out essentially as described above with respect to the cell free assays. Advantageously, the cell used

expresses predominantly the α_{1d}AR subtype. By way of example, the cell-based binding assay can be carried out by adding test compound (advantageously, bearing a detectable (e.g., radioactive or fluorescent) label), to medium in which the α_{1d}AR (or portion thereof) expressing cells are cultured, incubating the test compound with the cells under conditions favorable to binding and then removing unbound test compound and determining the amount of test compound associated with the cells.

As in the case of the cell-free assays, the cell-based assays can also take the form of competitive assays, as described above. For example, a compound known to bind α_{1d}AR (and preferably labelled with a detectable label) can be incubated with α_{1d}AR (or portion thereof) expressing cells in the presence and absence of test compound. The affinity of a test compound for α_{1d}AR can be assessed by determining the amount of known compound associated with the cells incubated in the presence of the test compound, as compared to the amount associated with the cells in the absence of the test compound.

A test compound identified in one or more of the above-described assays as being capable of binding to α_{1d}AR can, potentially, serve as an α_{1d}AR antagonist and therefore be suitable for use in the irritative symptom treatment method of the invention. To determine the specific effect of any particular test compound selected on the basis of its ability to bind α_{1d}AR, various assays can be used including IP assays (see Schwinn et al, J. Pharm. Exp. Ther. 272:134 (1995)) and bladder (e.g. human bladder) smooth muscle contraction assays (Ford et al, Mol. Pharm. 49:209 (1996)). Compounds suitable for use in treating irritative symptoms will be associated with antagonistic (inhibitory) effects in the IP assay and contraction inhibitory effects in the contraction assay.

In another embodiment, the invention relates to compounds identified using the above-described assays as being α_{1d}AR antagonist. The compounds identified in accordance with the above assays can be formulated as pharmaceutical compositions. Such compositions comprise the compound and a pharmaceutically acceptable diluent or carrier. The compound can be present in dosage unit form (e.g., as a tablet or capsule) or as a solution, preferably sterile, particularly when administration by injection is anticipated. The dose and dosage regimen will vary, for example, with the patient, the compound and the effect sought. Optimum doses and regimens can be determined readily by one skilled in the art.

In another embodiment, the invention relates to antibodies specific for α_{1d}AR, and antigen binding fragments thereof, including $F(ab)_2'$ or F(ab) fragments. The antibodies can be monoclonal or polyclonal and can be prepared using standard techniques. The antibodies can be used in α_{1d}AR purification protocols or the antibodies can be formulated as pharmaceutical compositions and used therapeutically as α_{1d}AR antagonists.

In yet another embodiment, the present invention relates to a gene therapy approach to treating irritative symptoms. In this embodiment, oligonucleotides (constructs) are used that, upon administration, result in the production of a molecule that down regulates production of α_{1d}AR. In a related embodiment, the present invention relates to α_{1d}AR antisense constructs and to a method of using same to treat irritative symptoms. Such constructs can be designed to target any of a variety of regions of the α_{1d}AR gene, including the encoding sequence (e.g., regions encoding the intracellular portion that interacts with G protein and participates in the signal transduction pathway) and the 5'-untranslated region.

Delivery of the above-described constructs can be effected using any of a variety of approaches, including installation into the bladder (e.g. via the uretha) and introduction into the cerebrospinal fluid. The constructs can also be administered systemically, in which case targeting can be effected using, for example, smooth muscle (e.g. bladder smooth muscle) specific promoters.

Effective vectors for use in the above-described gene therapy/antisense embodiments include viral vectors, such as retroviral vectors, adenoviral vectors and adenoassociated viral vectors. The constructs can also be present in association with a lipid, e.g. a liposome. (For details of antisense constructs and delivery systems, etc, see, for example, Wagner Nature 372:333 (1994).) The amount of construct to be administered will vary, for example, with the construct, the patient and the effect sought. One skilled in the relevant art can readily optimize the dose and treatment regimen.

In yet another embodiment, the invention relates to kits, for example, kits suitable for conducting assays described herein. Such kits can include α_{1d}AR, or portion thereof, for example, bound to a solid support. The kit can include an α_{1d}AR-encoding sequence, α_{1d}AR antisense construct or α_{1d}AR-specific antibody. The kit can include any of the above components disposed within one or more container means. The kit can further include ancillary reagents (e.g., buffers) for use in the assays.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

EXAMPLES

The following experimental details are relevant to the specific Examples that follow.

Tissue preparation. Full-thickness human bladder detrusor was obtained as discarded "normal" tissue adjacent to tumor specimens (n=1 radical cystectomy, n=12 radical cystoprostatectomy for transitional cell carcinoma of the bladder) with appropriate institutional approval. Each sample was inspected by a pathologist, and normal tissue confirmed. Detrusor smooth muscle was grossly teased from urothelial and serosal layers, snap frozen in liquid nitrogen within 30 minutes of excision, and stored at -70°C for later use. Whole rat bladder was obtained from euthanized male Sprague-Dawley rats (Charles River Laboratories; Wilmington, MA) with institutional animal care committee approval. Rat tissue was harvested within two minutes of death, snap frozen in liquid nitrogen, and stored at -70°C for later use.

Human detrusor and rat bladder membrane preparation. Human detrusor and rat whole bladder was minced over dry ice, and suspended in cold lysis buffer (5mM Tris HCl and 5mM EDTA, pH7.4) with protease inhibitors benzamidine (10mg/ml), leupeptin (5mg/ml), and soybean trypsin inhibitor (10mg/ml) (Sigma Chemical Company; St. Louis, MO). A lysate was prepared with a Polytron PT 3000 (Brinkmann; Westbury, NY) at 10,000 rpm for 10 seconds. After pelleting at 40,000 x g for 15 minutes (Sorvall SM24 rotor), membranes were suspended in cold resuspension buffer (150mM NaCl, 50mM Tris·HCl, 5mM EDTA, pH7.4) with protease inhibitors, and kept on ice for immediate use (or stored at -70°C for later use). Protein content was determined

using the bicinchoninic assay (BCA) with bovine serum albumin (BSA) standards (Pierce; Rockford, IL).

Radioligand binding. All mRNA and protein studies described were performed using detrusor from each patient described above (n=13). In order to conserve sample, and yet fully characterize α_1ARs in human detrusor, additional full saturation binding isotherms were generated in human detrusor samples from a subset of patients (n=5) using a buffer consisting of 150mM NaCl, 50mM Tris·HCl and 5mM EDTA, pH7.4, with protease inhibitors. Each reaction was performed in triplicate, in a total volume of 0.25 ml, including diluted human detrusor membranes (50 to 100 mg protein) and the α_1AR antagonist [^{125}I]HEAT (NEN Research Products-DuPont; Boston, MA) ranging in concentration from 2-900pM; nonspecific binding was measured in the presence of 1mM prazosin (Sigma). The reaction proceeded at 25°C for 45 minutes, and was terminated with five-fold dilution of ice-cold 50mM Tris HCl, pH7.4 buffer, followed by rapid filtration over GF/C filters using a Brandel harvester. Dried filters were then counted in a gamma counter. Specific binding was calculated by subtracting nonspecific binding from total binding. Saturation curves were fit with noniterative regression analysis using InPlot software (GraphPad; San Diego, CA). Total α_1AR density was then determined in each detrusor sample as described above, using a saturating concentration of [^{125}I]HEAT (300pM). Results are reported as mean±SEM to two significant figures.

To determine Ki values in human detrusor for α_1AR subtype discriminating ligands, competition binding was performed in triplicate in a total volume of 0.25ml using binding buffer (see saturation binding above). Human

detrusor membranes (50 to 100 μg protein) were incubated with a K_d concentration (120 pM) of the α_1AR antagonist [^{125}I]HEAT, and increasing concentrations (10^{-12} to 10^{-3}M) of the non-radiolabeled α_{1d}AR-selective ligand BMY7378 (Research Biochemicals International; Natick, MA). Reaction conditions were as described above. Curves were fit with noniterative regression analysis using InPlot software (GraphPad).

Preparation of RNA. Total RNA was extracted from human detrusor or rat whole bladder samples using the RNazol method (Tel-Test, Inc.; Friendswood, TX). RNA was quantitated using a spectrophotometer at 260/280 nm, and aliquoted into 20 mg samples for immediate use.

Human α_1AR cDNA constructs. The human α_{1a}AR probe consists of a 0.326kb (*Pvu*II/*Hind*III) fragment in pGEM-4Z (Promega Corporation; Madison, WI), corresponding to nucleotides 958-1283 of the cloned human α_{1a}AR cDNA (GenBank #L31774). The human α_{1b}AR probe consists of a 0.673kb (*Xho*I/*Bam*HI) fragment in pGEM-4Z (Promega), corresponding to nucleotides 94-766 of the cloned human α_{1b}AR cDNA (GenBank #L31773). The human α_{1d}AR probe consists of a 0.377kb (*Eco*RI/*Pst*I) fragment, corresponding to nucleotides 520-896 of the cloned human α_{1d}AR cDNA (GenBank #L31772). Figure 1 shows the location of each α_1AR subtype probe within a schematic of the encoded protein. The human cyclophilin probe consists of a 0.103kb (*Kpn*I/*Eco*RI) fragment in pTRI (Ambion, Inc.; Austin, TX), corresponding to nucleotides 38-140 of the cloned human cyclophilin gene (GenBank #X52856).

Labeling of RNA probes. Antisense single-stranded radiolabeled RNA probes were generated from linearized α_1AR cDNA constructs using RNA polymerase T7 (α_{1a}, cyclophilin) and SP6 (α_{1b}, α_{1d}) as described in the Promega Protocols and Applications Guide (Promega Corporation; Madison, WI). α_{1a}AR and α_{1d}AR cDNA constructs were linearized with *Eco*RI, and the α_{1b}AR cDNA construct was linearized with *Hind*III. ^{32}P-αUTP (NEN Research Products-DuPont) was incorporated into RNA probes at the time of probe synthesis. All probes were purified on a 5% polyacrylaminde gel (300V for 1.5hr); after exposure to film for 3 min, radiolabeled RNA probes were excised from the gel and passively eluted overnight into 400μl of RPA II kit (Ambion) elution buffer at 37°C.

RNase protection assays. RNase protection assays were conducted as previously described (Zinn et al, Cell 34:865-879 (1983)) with a few modifications. In brief, total RNA samples (20mg) were dissolved in 20ml of hybridization buffer containing >20-fold excess of radiolabeled probe (2 x 10^5 cpm/reaction for α_{1a}, α_{1b}, α_{1d}, and 1 x 10^5 cpm/reaction for cyclophilin), and incubated overnight at 55°C (α_{1a}, α_{1b}) and 65°C (α_{1d}, cyclophilin). To ensure specificity of the synthesized radiolabeled antisense human α_1AR subtype selective probes, RNase protection assays were performed in tandom with total RNA extracted from rat-1 fibroblast cells stably expressing each cloned human α_1AR subtype. As a negative control, RNase protection assays for each α_1AR subtype selective probe were performed in tandom with yeast tRNA samples and other non-hybridizing α_1AR subtypes. Antisense radiolabeled probe to the highly conserved region of the constitutively expressed human cyclophilin gene was also utilized as a control to ensure identical amounts of total RNA in each assay. The

final gel was exposed to X-Omat AR film (Eastman Kodak Company; Rochester, NY) for 24-72 hours.

α_1AR mRNA quantitation in human detrusor smooth muscle from RNase protection assays. In order to quantitate relative α_1AR subtype mRNA, each RNase protection assay final gel was exposed to PhosphorImager plates (Molecular Dynamics; Sunnyvale, CA) for 24 hours. Volume integration of specific protected radiolabeled bands for each mRNA resulting from hybridization products was corrected for background, normalized for cyclophilin signal, and expressed as arbitrary density units, using ImageQuant gel image-analysis software (Molecular Dynamics). α_1AR probes contained the following number of UTP sites for ^{32}P-αUTP incorporation: α_{1a} 88, α_{1b} 117, α_{1d} 51. Arbitrary density units were normalized to the lowest ^{32}P-αUTP incorporating probe (α_{1d}AR) and then expressed as a fraction (±SEM) of total α_1AR mRNA signal strength.

Polymerase Chain Reaction (PCR). RT-PCR was used to confirm expression of human detrusor α_1AR subtypes (to ensure low concentrations of a subtype were not missed in human bladder) and to compare α_1AR subtype mRNA expression in rat whole bladder with previously published rat data (Walden et al, J. Urol. 157:1032-1038 (1997), Scofield et al, J. Pharmacol. Exper. Ther. 275:1035-1042 (1995)). Human and rat α_1AR subtype primers were synthesized at Duke University Medical Center. Reverse transcription of 1mg of DNase-treated human detrusor or rat bladder RNA was performed in triplicate in a 20ml reaction mixture containing 5mM $MgCl_2$, 1mM each of dATP, dCTP, dGTP, and dTTP, 10mM Tris·HCl, 50mM KCl, 2ml DEPC treated water, 2.5mM random hexamers, 1 unit of RNase inhibitor, and 2.5 units of MuLV Reverse

Transcriptase (Perkin Elmer; Foster City, CA); simultaneous control samples not treated with reverse transcriptase were used to rule out amplification of genomic DNA. Reverse transcriptase reactions were run for 60 min at 42°C, 5 min at 95°C, and 10 min at 4°C. Each α_1AR mRNA subtype was amplified by PCR in triplicate in a 100ml reaction containing 50mM KCl, 10mM Tris·HCl, pH8.3, 2mM $MgCl_2$, 200mM each of dATP, dCTP, dGTP and dTTP, 15pM of sense and antisense primer, 5% DMSO, and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer). PCR reactions were performed in a DeltaCycler II™ temperature cycler (ERICOMP; San Diego, CA). The following conditions were established for all three rat primer sets: one denaturation cycle for 3 minutes at 95°C, 35 cycles of 1 min at 95°C, 1 min annealing at 58°C, and a 1 min extension at 72°C. The following conditions were established for all three human primer sets: one denaturation cycle for 3 minutes at 95°C, 35 cycles of 1 min at 95°C, 1 min annealing at 60°C for α_{1a} and α_{1b}, and 68°C for α_{1d}, and a 1 min extension at 72°C. A final extension cycle was performed for 10 min at 72°C. Reaction mixtures were then cooled at 4°C. 10ml of each PCR product was separated by gel electrophoresis in 0.8 % agarose. Since PCR experiments were only confirmatory in nature by design, exact quantitation (requiring competitive PCR) was not performed. However, to ensure that any statement regarding relative mRNA levels is appropriate, it is important to note that conditions described above (e.g. different annealing temperatures) were chosen after extensive preliminary analysis with each primer set to ensure optimal amplification conditions with similar primer product efficiency. Equality of reverse transcription efficiency for products was checked using equal concentrations of starting control cDNA; these reactions also served as a positive control for use of correct primer sets. Thirty-five cycles of amplification was chosen since it is at the upper end of the linear

amplification range for all six primer sets (α_1AR mRNAs are rare at baseline in many human tissues and in our hands 40 cycles of amplification is where the curve becomes non-linear).

EXAMPLE 1

Human Patient Population

Human detrusor smooth muscle was obtained from male (n=12) and female (n=1) patients. Patient age ranged from 56 to 76 years old (mean = 59.6). Significant past medical history included tobacco abuse, coronary artery disease, hypertension controlled with α_1AR or βAR antagonists (n=3), and a history of BOO (n=2) necessitating previous transurethral resection of prostate. Comparison of results from patients with hypertension and/or BOO (n=5) suggests medical history did not affect the results. A larger study would be required to make any definitive statement in this regard.

EXAMPLE 2

α_1AR Ligand Saturation Binding

Pharmacological characteristics of α_1ARs in human detrusor include a Kd for the radiolabeled α_1AR antagonist [^{125}I]HEAT of 130±1.9 pM, similar to that reported for cells stably expressing the cloned human α_{1a}AR subtype ((Schwinn et al, J Pharmacol. Exper. Ther. 272:134-142 (1995)). A representative saturation binding isotherm is shown in Figure 2. Total α_1AR density as measured by saturation binding in human detrusor membrane preparations with the α_1AR

antagonist [^{125}I]HEAT is 6.3±1.0 fmol/mg protein (mean±SEM, range 2.7-9.0, n=13). Although low (with corresponding high non-specific binding of 70-80% as expected), α_1AR expression is reproducible and consistent within and between patients.

EXAMPLE 3

Identification and Quantification of the α_1AR mRNA Subtypes in Human Bladder Detrusor

In order to determine which α_1AR subtypes are present in human detrusor, molecular approaches were chosen due to their sensitivity and specificity. To ensure specificity of the synthesized radiolabeled antisense human α_1AR subtype selective probes, RNase protection assays were performed simultaneously with total RNA extracted from rat-1 fibroblast cells stably expressing each cloned human α_1AR subtype. Each α_1AR subtype specific probe protects a single predominant fragment of predicted size without cross-hybridization (Figure 3, positive control cells); a lack of cross-hybridization between subtypes with each probe (Price et al, Mol. Pharmacol. 46:221-226 (1994)). As was previously demonstrated a further negative control, RNase protection assays for each α_1AR subtype selective probe were performed in tandom with yeast tRNA samples, where no hybridization is demonstrated (Figure 3, tRNA lane). Human detrusor contains α_{1d}AR>α_{1a}AR mRNA, but no α_{1b}AR mRNA in every patient studied (n=13; Figure 3 shows representative results from patients number 1 through 5). This data, when corrected for background, normalized for cyclophilin content, and corrected for probe ^{32}P-αUTP incorporation, reveals that α_{1d}AR mRNA constitutes 66±4.8% and α_{1a}AR mRNA 34±4.8% of the total α_1AR mRNA in human detrusor.

EXAMPLE 4

Confirmation of α_1AR Subtype mRNA in Human Detrusor and Comparison with Rat Whole Bladder using RT-PCR

In order to confirm results from RNase protection assays and to compare with another frequently used animal model (rat), α_1AR subtype expression was examined using RT-PCR in each patient. Primer nucleotide sequences, melting temperatures (T_m), and primer positions relative to the cDNA sequence are shown in Table 1 and Table 2; these primers do not span an intron.

TABLE 1. Oligonucleotide primers used for rat α_1AR subtype RT-PCR.

Rat α_1AR primers	nucleotide sequences 5' 3'	T_m	primer position relative to cDNA
α_{1a}AR sense	GTAGCCAAGAGAAAGCCG	62°C	628-647
α_{1a}AR antisense	CAACCCACCACGATGCCCAG	66°C	839-820
α_{1b}AR sense	GCTCCTTCTACATCCCGCTCG	68°C	629-649
α_{1b}AR antisense	AGGGGAGCCAACATAAGATGA	62°C	928-908
α_{1d}AR sense	CGTGTGCTCCTTCTACCTACC	66°C	759-779
α_{1d}AR antisense	GCACAGGACGAAGACACCCAC	68°C	1062-1042

TABLE 2. Oligonucleotide primers used for human α_1AR subtype RT-PCR.

Human α_1AR primers	nucleotide sequences 5' 3'	T_m	primer position relative to cDNA
α_{1a}AR sense	ATCATCTCCATCGACCGCTACA	66°C	355-376
α_{1a}AR antisense	TCACTTGCTCCGAGTCCGACTT	68°C	697-676
α_{1b}AR sense	GCTCCTTCTACATCCCTCTGG	68°C	629-649
α_{1b}AR antisense	AGGGTAGCCAGCACAAGATGA	67°C	928-908
α_{1d}AR sense	ACCACGCGCAGCCTCGAGGCAGGC	84°C	850-873
α_{1d}AR antisense	GAGCGAGCTGCGGAAGGTGTGGCC	82°C	999-976

Although RNase protection assays are considered the "gold standard" for quantitating mRNA present in a given tissue, this approach is not as sensitive as PCR, therefore very small amounts of mRNA can be missed in a RNase protection assay but demonstrated by PCR. As shown in Figure 4, RT-PCR performed on human detrusor total RNA demonstrates the presence of α_{1a}AR and α_{1d}AR mRNA, and lack of α_{1b}AR mRNA, consistent with data from the RNase protection assays. Of note, α_{1d}AR mRNA accounts for approximately 60-70% of total α_1AR mRNA in human detrusor with α_{1a}AR mRNA accounting for 30-40%, again confirming the RNase protection assay results. Species heterogeneity (human versus rat) of α_1AR subtype mRNA expression has been previously reported for many tissues (Price et al, Mol. Pharmacol. 46:221-226 (1994), Price et al, Mol. Pharmacol. 45:171-175 (1994)). Indeed, as seen in Figure 4, RT-PCR performed on pooled rat bladder total RNA demonstrates the presence of all three α_1AR mRNAs in roughly equal concentrations in rat.

EXAMPLE 5

Determination of α_1AR Subtype Expression at a Protein Level

Competition analysis was used to determine α_1AR subtype expression at a protein level in human detrusor. Since molecular studies demonstrate a predominance of α_{1d}AR mRNA, the α_{1d}AR-selective compound BMY7378 was used in these studies. As graphically represented in Figure 5, a two-site fit was evident in every patient studied (n=13), with high affinity binding predominating (high pK_i = 8.6±0.2 [66±3.1% total] vs. low pK_i = 4.9±0.2 [35±3.1% total]) (Table 3).

TABLE 3. Results from competition binding experiments utilizing membranes from rat-1 fibroblasts stably transfected with each α_1AR subtype (controls) and human detrusor (n=13). Since no α_{1b}AR was found in human detrusor by RNase protection assays and RT-PCR, one versus two site fit of the data was utilized.

	BMY7378 (pK_i)				
	α_{1a}AR	α_{1b}AR	α_{1d}AR	% High	% Low
Human detrusor	4.9±0.2	—	8.6±0.2	66±3.1	35±3.1
Control α_{1a}	4.8±0.1	—	—		
Control α_{1b}	—	5.1±0.3	—		
Control α_{1d}	—	—	8.5±0.1		

* * *

All documents cited above are hereby incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. A method of treating irritative symptoms of bladder or lower urinary tract disease comprising administering to a patient in need of such treatment an amount of an α_{1d}-adrenergic receptor (α_{1d}AR) antagonist sufficient to effect said treatment.

2. The method according to claim 1 wherein said patient suffers from a disease of the bladder.

3. The method according to claim 1 wherein said patient suffers from a disease of the lower urinary tract.

4. The method according to claim 1 wherein the patient is a female.

5. The method according to claim 4 wherein said α_{1d}AR antagonist is specific for α_{1d}AR.

6. The method according to claim 1 wherein the patient is male.

7. The method according to claim 6 wherein said α_{1d}AR antagonist is an α_{1d}AR antagonist and an α_{1a}AR antagonist.

8. The method according to claim 1 wherein said symptoms are selected from the group consisting of frequent urination, urgent urination, nocturia and unstable bladder contractions.

9. A method of treating irritative symptoms of bladder or lower urinary tract disease comprising administering to a patient in need of such treatment an effective amount of a construct comprising an oligonucleotide that inhibits production of α_{1d}AR or that encodes a molecule that inhibits production of α_{1d}AR.

10. The method according to claim 9 wherein said construct is an antisense construct.

11. A method of screening a test compound for its ability to bind α_{1d}AR comprising incubating said test compound with α_{1d}AR, or portion thereof, and determining the amount of said test compound bound to said α_{1d}AR, or portion thereof.

12. The method according to claim 11 wherein said portion comprises the transmembrane portion of α_{1d}AR.

13. The method according to claim 11 wherein said test compound bears a detectable label.

14. The method according to claim 11 wherein said α_{1d}AR, or portion thereof, is present in a cell membrane.

15. The method according to claim 14 wherein said membrane is the membrane of an intact cell.

16. The method according to claim 15 wherein said cell is a eucaryotic cell.

17. The method according to claim 15 wherein said cell is a cell that has been engineered to express or over-express said α_{1d}AR, or portion thereof.

18. The method according to claim 11 wherein said test compound is incubated with said α_{1d}AR, or portion thereof, in the presence of an agent known to bind to α_{1d}AR, or portion thereof, and the amount of said test compound that binds to said α_{1d}AR, or portion thereof, is determined indirectly by determining the amount of said agent that binds to said α_{1d}AR, or portion thereof.

19. The method according to claim 18 wherein said agent bears a detectable label.

20. The method according to claim 18 wherein said agent is BMY7378 or tamsulosin.

21. A compound identified using the method according to claim 11.

22. A composition comprising the compound according to claim 21, and a pharmaceutically acceptable carrier.

23. A kit comprising α_{1d}AR, or portion thereof, bound to a solid support.

24. A kit comprising α_{1d}AR, or portion thereof, labeled with a detectable label.

25. A cell engineered to express or over-express α_{1d}AR, or portion thereof.

26. An antibody specific for α_{1d}AR.

27. An antisense construct comprising an oligonucleotides, operably linked to a promoter, the transcript of said oligonucleotide being complementary to α_{1d}AR mRNA.

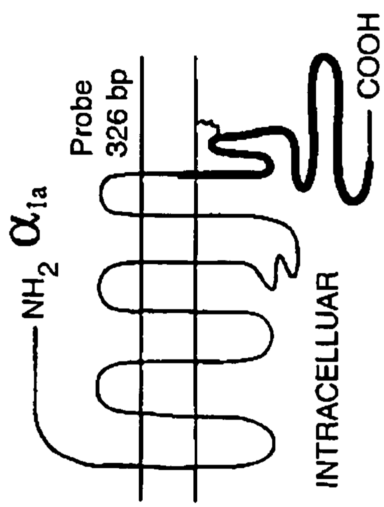


Fig.1A

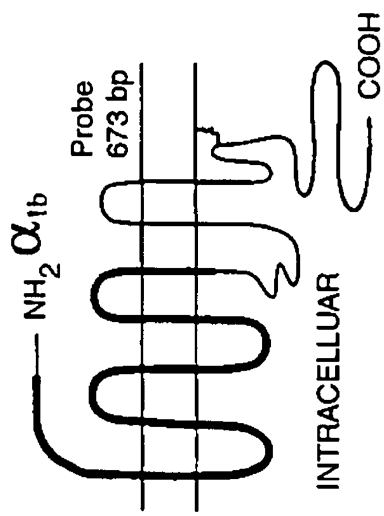


Fig.1B

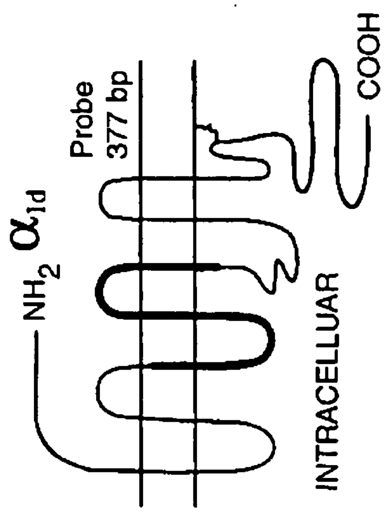


Fig.1C

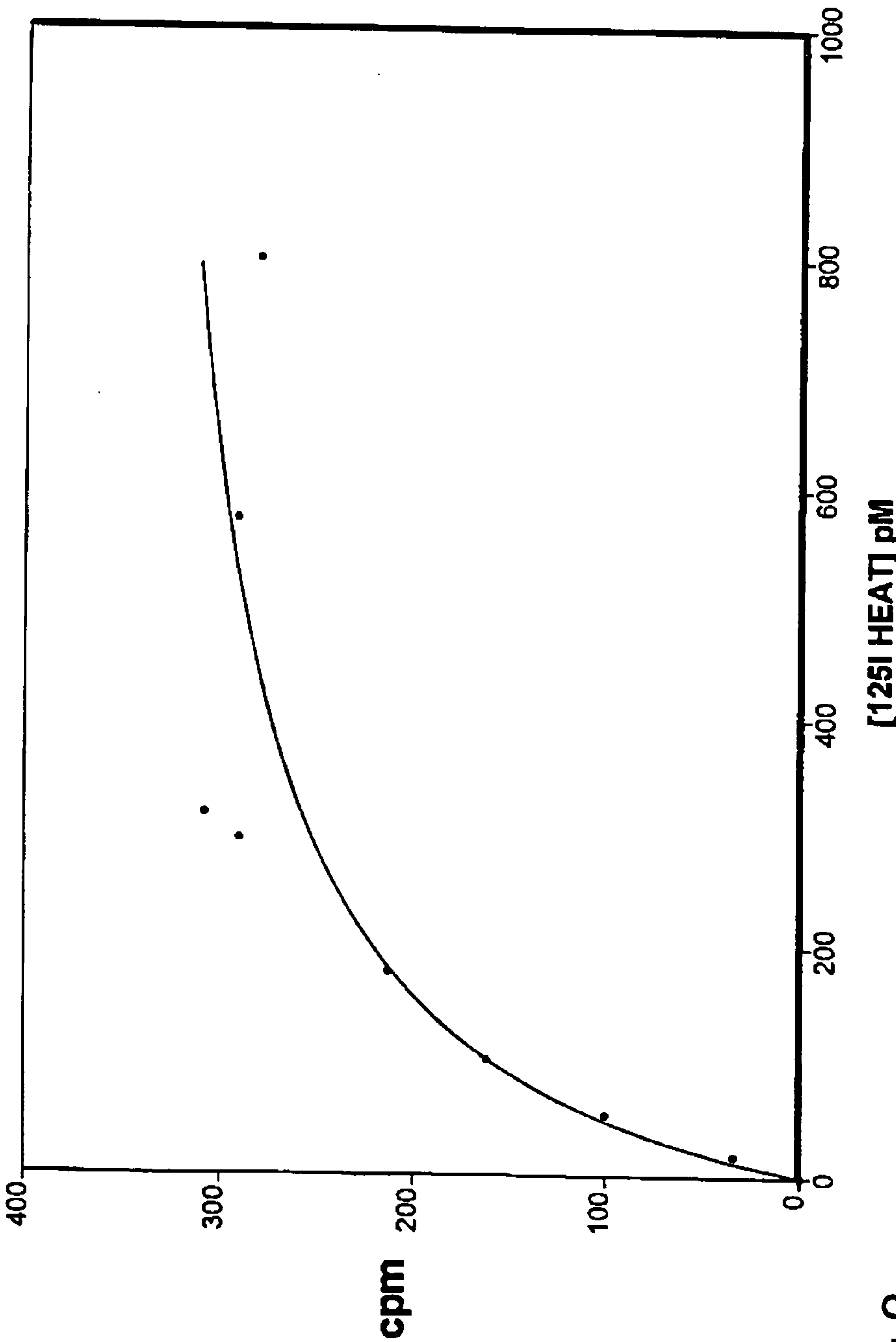


Fig.2

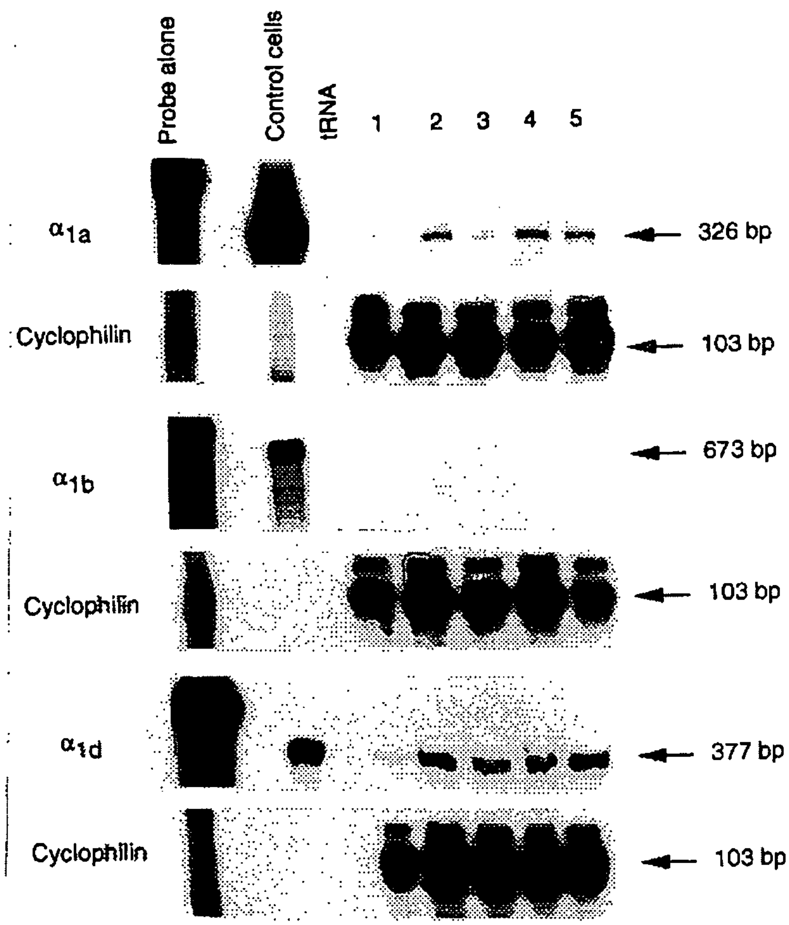

Probe alone
Control cells
tRNA
1
2
3
4
5
α_{1a}
326 bp
Cyclophilin
103 bp
α_{1b}
673 bp
Cyclophilin
103 bp
α_{1d}
377 bp
Cyclophilin
103 bp

Fig.3

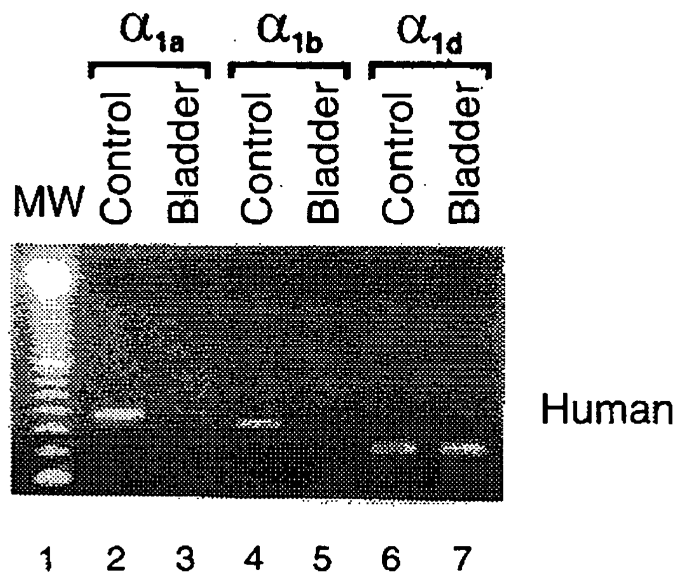

α_{1a}
α_{1b}
α_{1d}
MW
Control
Bladder
Control
Bladder
Control
Bladder
Human
1 2 3 4 5 6 7

Fig.4A

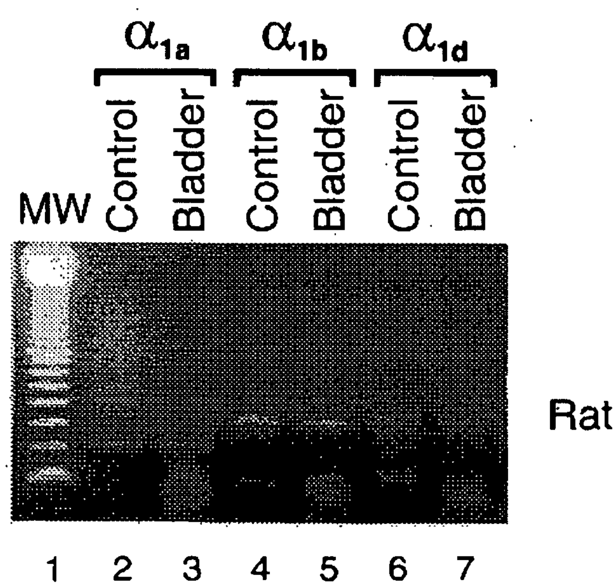
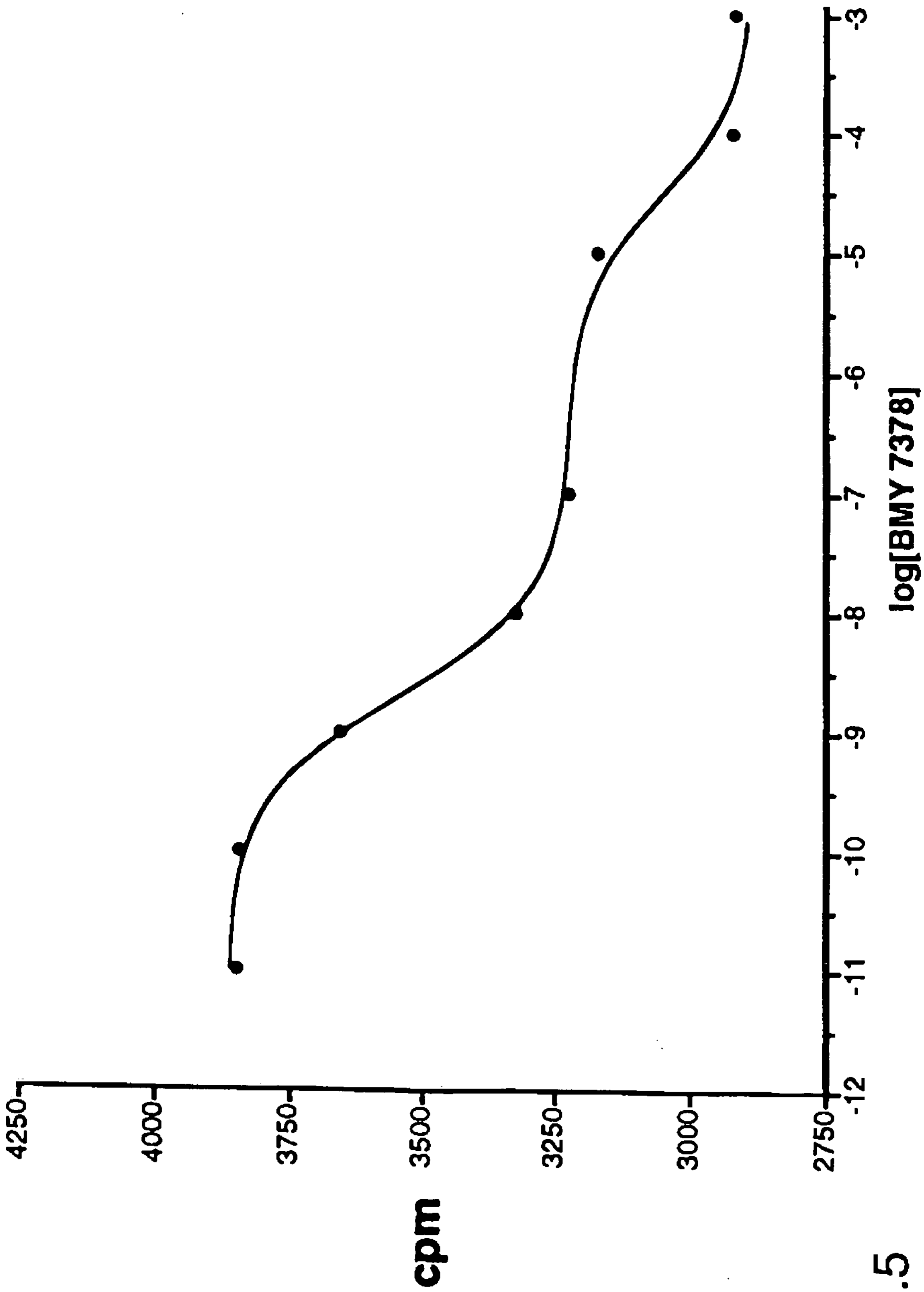

α_{1a}
α_{1b}
α_{1d}
MW
Control
Bladder
Control
Bladder
Control
Bladder
Rat
1 2 3 4 5 6 7

Fig.4B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4000
3750
3500
3250
3000
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-11
-10
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-8
-7
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-5
-4
-3
log[BMY 7378]

Fig.5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09846

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12N 15/85; C07K 14/00, 16/00; A61K 48/00; C12Q 1/68
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.1; 424/130.1; 435/6, 7.1, 7.93, 69.5; 514/44; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRAHAM et al. Alpha 1-Adrenergic Receptor Subtypes. Circ. Res. 1996, Vol.78, No.5, pages 737-749, see entire document.	1-27
A	DOCHERTY, J.R. Subtypes of functional alpha1- and alpha2-adrenoceptors. Euro. J. Pharmacology. 1998, Vol.361, No.1, pages 1-15, see entire document.	1-27
A,P	Malloy et al. Alpha1-Adrenergic Receptor Subtypes In Human Detrusor. J. Urology. September 1998, Vol.160, pages 937-943, see entire document.	1-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 21 JULY 1999	Date of mailing of the international search report 09 SEP 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANDREW WANG D. Lawrence Tarr Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/09846

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	RICHARDSON et al. Pharmacology of Tamsulosin: Saturation-Binding Isotherms and Competition Analysis Using Cloned alpha1-Adrenergic Receptor Subtypes. The Prostate. 1997, Vol. 33, pages 55-59, see entire document.	1-8, 11-17, 21-25 ---- 18-20, 26, 27
X - Y	MICHEL et al. Alpha-Adrenergic regulation of human renal function. Fundam. Clin. Pharmacol. 1996, Vol. 10, pages 493-503, see entire document.	1-8 ---- 9-27
Y	DOCHERTY et al. The alpha-Adrenoreceptor-Mediated Actions of Chloroethylclonidine. Gen. Pharmacology. 1997, Vol. 28, No. 2, pages 197-201, see entire document.	1-27
X - Y	ANDERSSON et al. Prostatic alpha1-Adrenoceptors and Uroselectivity. The Prostate. 1997, Vol. 30, pages 202-215, see entire document.	1-8 ---- 9-27

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/387.1; 424/130.1; 435/6, 7.1, 7.93, 69.5; 514/44; 536/23.1, 24.5